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A Mouse Monoclonal Antibody Specific for an Allotypic Determinant of the *Igh*^{a} Allele of Murine IgM: Genetic and Functional Analysis of Igh-6a Epitopes Using Anti-IgM Monoclonal Antibodies

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ABSTRACT

An IgG_1 mouse monoclonal antibody (MAb) specific for a mouse IgM allotypic determinant in the a, c, f, g, h, and j haplotypes was derived from a fusion of SP2/0-Ag14 mouse myeloma cells with C57BL/6 mouse spleen cells (*Igh-C*^{b}) immune to TC31, a MAb of the IgM^a allotype. MAb from one hybridoma derived from this fusion (designated DS1) was demonstrated to bind in an ELISA to immunoglobulin bearing the IgM^a allotype (TC31, MOPC104E), but not to immunoglobulin bearing the IgM^b allotype (C.BPC112). Fluorescein-conjugated DS1 was shown to bind to the surface of BALB/cByJ splenic B cells, but was shown to have negligible binding on C57BL/6J cells. Similarily, DS1-conjugated Sepharose beads were able to stimulate in vitro proliferation of BALB/c, but not C57BL/6 splenic B cells. DS1 was unable to bind to spleen cells from BALB/c allotype congenic strains, BAB/14 (*Igh-C*^{b}) and C.AL-20 (*Igh-C*^{o}), demonstrating that DS1 recognizes a determinant under the control of a gene linked to the *Igh-C* gene complex. Using sera from recombinant inbred lines, the determinant defined by DS1 was shown to be linked to the *Igh-1* locus. Furthermore, the determinant was localized to the CH1 domain of the μ heavy chain. Sera from BALB/cByJ, NMRI, CBA/J, SEA/GnJ, RIIIs/J, and CE/J mouse strains were shown to bind to DS1 in an ELISA, while sera from A/J, SJL/J, NZB/B1NJ, AKR/J, C57BL/6J, and C57BL/10SnJ mouse strains did not bind to DS1. From these data we propose that DS1 is reactive with specificity Igh-6.1, which was originally defined by an allotypic antiserum developed by Black *et al.* (Immunogenetics 7:213, 1978).

INTRODUCTION

The availability of polyclonal and monoclonal antibody (MAb) reagents directed at isotypic or allotypic determinants of membrane bound IgM on mouse B lymphocytes has enabled various studies of B lymphocytes, including studies demonstrating the role of membrane IgM in the activation (1-4) and differentiation of B lymphocytes (5), and in phenotyping of immune responses of transgenic mice (6). Allotypic determinants on mouse IgM were originally defined using allo-antisera raised by immunization with spleen cells or with antigen-antibody complexes (7, 8). These antisera defined the locus, *Igh-6*, which encodes the constant region of the μ heavy chain for mouse IgM (7). These antisera also made it possible to define the a, b, and e alleles for the *Igh-6* locus and four antigenic specificities, termed 6.1, 6.2, 6.3 and 6.4 (8, reviewed in 9).

With the advent of MAb technology, MAb reagents with greater specificity for the μ chain allotypic determinants have been developed. Two rat MAb, 331.12 (10,11), and Bet 1 (12) defined specificity Igh-6.5, which is present in BALB/c, DBA/2, and CBA/J, and weakly detected in C57BL/6, AKR, and NZB. A mouse MAb, AF6-78.25 (13) defined a new specificity, Igh-6.6, present in mouse strains of the *Igh-C*^{b,d,n} haplotypes; and MB86 (14), was reported as a mouse MAb against specificity Igh-6.4, which is present in C57BL/6, AKR, and NZB, and weakly present in A and AL strains. Only one other mouse MAb, RS-3.1, has recently been described, which was shown to react with IgM from BALB/c, DBA/2 and CBA/J, but not with C57BL/6, A, AKR, or NZB (15).

This communication is an extension of our previous report (16) on a mouse MAb, named DS1, with anti-allotypic specificity similar to that of RS-3.1. The reactivity of DS1 with the IgM^a allotype, but not with the IgM^b or IgM^n allotypes was originally defined by the alloantisera (C57BL anti-CBA spleen) developed by Black, et. al. (8) and defined as specificity Igh-6.1. We have further defined the specificity Igh-6.1 as occurring in haplotypes a, c, f, g, h and j, but not in haplotypes b, d, e, n and o, and have provided experimental evidence for the localization of this determinant to the CH1 domain of the μ heavy chain.

MATERIALS AND METHODS

Mice and Mouse Sera

A/J, AKR/J, BALB/cJ, BALB/cByJ, C57BL/6J, C57BL/10SnJ, CBA/J, CByB6F1/J, CE/J, DBA/2J, NZB/BlNJ, RIIIs/J, and SEA/GnJ mice, and the BxD and AKxL recombinant inbred (RI) lines were purchased from The Jackson Laboratory, Bar Harbor, ME. C.B20, C.AL-20, and BAB/14 mice were obtained from Dr. Michael Potter, NCI, NIH, through NCI contract no. CB05596-17 maintained with Hazelton Laboratories America, Inc., Rockville, MD. C.B20 and BAB/14 are BALB/c congenic mice bearing the *Igh-C*^{b} locus of C57BL/6, and C.AL-20 is a BALB/c congenic mouse bearing the *Igh-C*^{a} locus of AL/N. NMRI mice were obtained from the Laboratory Animal Sciences Division, Naval Medical Research Institute. Mice were used between the ages of 8 and 24 weeks. Sera from AL/N mice were generously provided by Mr. Bill Humphrey, NIAID, NIH, Bethesda, MD.

Antibodies

TC31 (a gift of Dr. Darrell Galloway, Ohio State University, Columbus, OH) is a hybridoma cell line derived from NMRI mice, which produces IgM, kappa monoclonal antibodies specific for exotoxin A (17). The hybridoma was grown as an ascites in sublethally irradiated (400 rad) BALB/cJ mice. Myeloma proteins MOPC104E (IgM, lambda) and CBPC.112 (IgM, kappa), MOPC245 (IgG$_1^b$, kappa), and MOPC21 (IgG$_1^a$, kappa) were produced as ascites from myeloma cell lines which were provided by Dr. Michael Potter. MOPC21 (Igh-1a) and MOPC245 (Igh-1b) served as allotype matched contros for DS1 and AF6-78.25, respectively. The mouse IgM monoclonals and myeloma proteins were purified from the ascitic fluid by preparative centrifugation at 147,000 x G for 16 h, followed by chromatography on a Sepharose 6B column in a 0.01 M borate buffered saline, pH 8.4.

Culture supernatants of rat anti-mouse IgM monoclonals R33, B76, IM41, and 2911, which are specific for the four domains of the μ heavy chain (18), and 331.12 (10,11) were kindly supplied by Dr. Fred Finkelman, Uniformed Services University of the Health Sciences, Bethesda, MD. Rat anti-mouse IgM monoclonal, Bet 1, anti-Igh-6.5 (12), was purified from culture supernatants by protein A affinity chromatography. Rat anti-mouse μ-chain monoclonal Bet 2 (12) was purified from culture supernatants by precipitation with 50% saturated ammonium sulfate (SAS) followed by DEAE-cellulose ion exchange chromatography in0.01 M Tris buffer, pH 8.5. AF6-78.25 (13), a hybridoma producing a mouse IgG$_1$, kappa monoclonal specific for the b haplotype of mouse IgM was purified from ascites by SAS precipitation and passage through a DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column in 0.01 M Tris buffer pH 8.0.

Fluorescein-conjugated-MOPC245 (FL-MOPC245), FL-DS1, and FL-AF6-78.25 were prepared by incubating the purified proteins at 10mg/ml, in 0.15 M NaCl containing 0.07M $NaHCO_3/Na_2CO_3$ buffer, pH 9.5, with fluorescein isothiocyanate

isomer I on Celite 10% (Calbiochem, La Jolla, CA) at a ratio of 4:1 (w:w) at room temperature for 1 h followed by centrifugation and dialysis or filtration through a column of Sephedex G-25. The F:P ratios were 3.1, 5.6 and 4.1, respectively. The FL-conjugated-goat anti-mouse IgG was purchased from Cappel Division of Organon Teknika, Durham, NC. The biotinylated MAR18.5 (19) was a gift of Dr. James J. Kenny, Program Resources,Inc., NCI-FCRF, Frederick, MD. The AP-conjugated avidin was purchased from Cappel.

Hybridoma Production and Tissue Culture

C57BL/6J mice were immunized with purified TC31 after the method of Lieberman and Humphrey (20), which included an initial s.c. and footpad injection with 75 μg of TC31 in CFA, followed by a second s.c. and footpad injection of 75 μg in IFA on day 4 and 75 μg in saline s.c. on day 7, and at weekly intervals for 9 weeks. The animals were rested for 8 weeks and given an i.v. injection of 75 μg TC31 in saline 3 days before use for fusion. The spleens were removed, dispersed into a single cell suspension, and fused with SP2/0-Ag-14, a nonsecreting 8-azaguanine resistant hybridoma cell line (21), using the method of Oi and Herzenberg (22), in the presence of 50% polyethylene glycol, mol. wt. 1000 (Sigma). After three weeks of incubation in 96-well microtiter plates (Costar, Cambridge, MA), 100 μl samples of supernatants were removed and tested for antibody binding activity to TC31 by an ELISA on TC31 coated plates. The positive hybrid cell lines were cloned by limiting dilution on feeder layers of CByB6F1/J thymocytes in 96-well plates.

Mouse spleen cells were cultured and assayed for a proliferative response as previously described (23). Monolconal antibodies were conjugated to cyanogen bromide activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO), previously washed in 10^{-3} M HCl, at a ratio of 1 mg antibody protein to 1 ml packed volume of beads, in a 0.1 M $NaHCO_3$ buffer, pH 8-9.

ELISA

The method originally described by Engvall and Perlman (24) was adapted for use in 96-well microtiter plates (Immulon 2, Dynatech Laboratories Inc., Chantilly, VA) (25). Microwells were coated overnight with 100 μl of purified TC31 or other antibody preparations at 5 μg/ml in a 0.01 M Na_2CO_3 buffer, pH 9.6, containing 0.2% NaN_3, and washed with a washing working buffer (WWB, borate buffered saline, pH 9.6, with 0.01% merthiolate, and 0.5% Tween 20 (Sigma) containing 1% crystallized and globulin-free bovine serum albumin (BSA, Sigma) using a plate washer (Dynawasher-II, Dynatech). The wells were filled with 100 μl of sample supernatant or ascites diluted in working buffer (WB, same as WWB except containing 5% BSA), and incubated for 2 h at 37°C. The plates were washed with WB, and 100 μl per well of alkaline phosphatase (AP)-conjugated, affinity-purified goat anti-mouse IgG, specific for Fc fragment (Cappel) was added and incubated for 2 h. The plates were washed with WWB and then with a diethanolamine buffer (94% diethanolamine, 0.01% $MgCl_2 \cdot H_2O$, 0.01% merthiolate, pH 9.8). The p-nitrophenylphosphate substrate (Sigma 104 Tablets, Sigma) dissolved in diethanolamine buffer at 1 mg/ml was added at 100 μl per well and incubated for 20 min at 37°C. The color reaction was stopped by addition of 100 μl of 2 N NaOH to each well. Plates were read for optical density (OD) at 405 nm in a Dynatech MR600 ELISA Reader.

In experiments testing for the ability of various anti-IgM antibodies to block the binding of DS1 to TC31, TC31 plates were first incubated for 2 h with the inhibitor sera or hybridoma supernatants at various dilutions in duplicate wells. The plates were subsequently washed with WWB and an optimal concentration of DS1 ascites was added to each inhibited well and also to wells which had only received WB in the first incubation. Detection of DS1 binding was followed as described above. The average amount of binding of the duplicate wells was calculated as a percentage of total binding of DS1 without any inhibitor protein present.

In experiments designed to test for the reactivity of DS1 or AF6-78.25 with serum IgM from various mouse strains, a 1:1500 dilution of DS1 or AF6-78.25 ascites was incubated with an equal volume of various dilutions of the mouse sera

at 37°C for 2 h and overnight at 4°C. The mixtures were then plated on ELISA plates coated with either TC31 or C.BPC112, for measuring DS1 or AF6-78.25, respectively, which had not absorbed to IgM in the serum. Binding of nonabsorbed monoclonal to the plates was measured by a final incubation with an AP-conjugated, affinity-purified goat anti-mouse IgG (Fc fragment specific) (Cappel). The percent inhibition by each serum was calculated as (OD of the inhibited mixture ÷ OD of the control binding of the MAb after incubation with WB) x 100%. To control for the presence of IgM in each inhibitor serum, serum dilutions were also incubated with an AP-conjugated goat anti-mouse μ chain specific antibody (TAGO, Inc., Burlingame, CA) and tested for their ability to inhibit the binding of the goat anti-μ to TC31 coated plates.

Analysis of Membrane Binding Using the Fluorescence Activated Cell Sorter (FACS)

Spleens were gently teased into single cell suspension in HBSS without phenol red (Grand Island Biological Co., Grand Island, NY). Contaminating erythrocytes were removed by resuspending the cells in 5 ml of an ammonium chloride solution (M.A. Bioproducts, Columbia, MD) for 1 min and then washing 3 times in HBSS containing 5% FCS (HyClone Laboratories, Inc., Logan, UT) and 0.1% NaN_3 (HBSS + FCS). Fifty μl aliquots (10^6 cells) were prepared in 5 ml test tubes and 50 μl of an appropriate dilution of the FL-conjugated antibody in HBSS + FCS was added. The cells were incubated for 30 min on ice and then washed twice in the HBSS + FCS. The cells were resuspended in 1.0 ml of HBSS + FCS and analyzed by flow microfluorimetry on a FACS II (Becton Dickinson, Sunnyvale, CA) modified with a five-decade logarithmic amplifier. For each histogram, 10,000 viable cells were analyzed. The abscissa of each histogram is the fluorescence intensity expressed in arbitrary units. Histograms of cells labeled with anti-IgM antibodies were compared with the histograms of cells labeled with an irrelevant antibody (FL-MOPC 254) and/or unstained preparations.

RESULTS

Production and Characterization of DS1

C57BL/6 mice were immunized with TC31 (IgM^a, kappa), a MAb specific for *Pseudomonas aeruginosa* exotoxin A. These mice were shown to be producing anti-allotypic antibodies, as demonstrated by the reactivity of their serum antibodies with TC31 and MOPC104E (IgM^a, lambda myeloma protein), but not with C.BPC112 (IgM^b, kappa myeloma protein) in an ELISA (data not shown). When spleen cells from these mice were fused with SP2/0 myeloma cells, a clone was derived which reacted strongly with TC31 and MOPC 104E, but not with C.BPC112. DS1 culture supernatant and ascites (from (BALB/cByJ x C57Bl/6)F_1 (CByB6F1/J) mice) both tested positive for binding in an ELISA on TC31 or MOPC104E, however, no binding was detected on C.BPC 112 (Fig. 1). As a control, AF6-78.25, a monoclonal antibody reactive with mouse IgM^b allotype (11), showed reactivity on C.BPC112 coated plates, but not on TC31 or MOPC104E. Since antibody produced by this clone bound to purified preparations of two IgM^a allotype antibodies, having different idiotype, but did not bind to IgM^b allotype bearing antibody, it was concluded that this clone was specific for IgM^a allotypic determinants. The clone was isotyped as an IgG_1 and named DS1.

Allotypic Specifications of DS1

The allotypic specificities recognized by DS1 were further determined by using a panel of normal sera from mouse strains of each of the known haplotype designations. Dilutions of these sera were tested for their ability to be bound by DS1 or control MOPC245 coated ELISA plates. The data in Table 1 corrected for non-specific binding on MOPC245 show that DS1 bound serum from the Igh^a (BALB/cByJ and NMRI), Igh^c (DBA/2J), Igh^f (CE/J), Igh^g (RIIIs/J), Igh^h (SEA/GnJ), and Igh^j (CBA/J) haplotypes. Sera from Igh^b (C57BL/6J, C57BL/10SnJ, C.B20 and SJL/J), Igh^d (AKR/J), Igh^e (A/J), Igh^n (NZB/BlNJ), and Igh^o (AL/N) haplotypes were not bound by DS1. These data were also supported by an experiment (data not shown) which used these sera to inhibit the binding of DS1 to TC31 coated ELISA

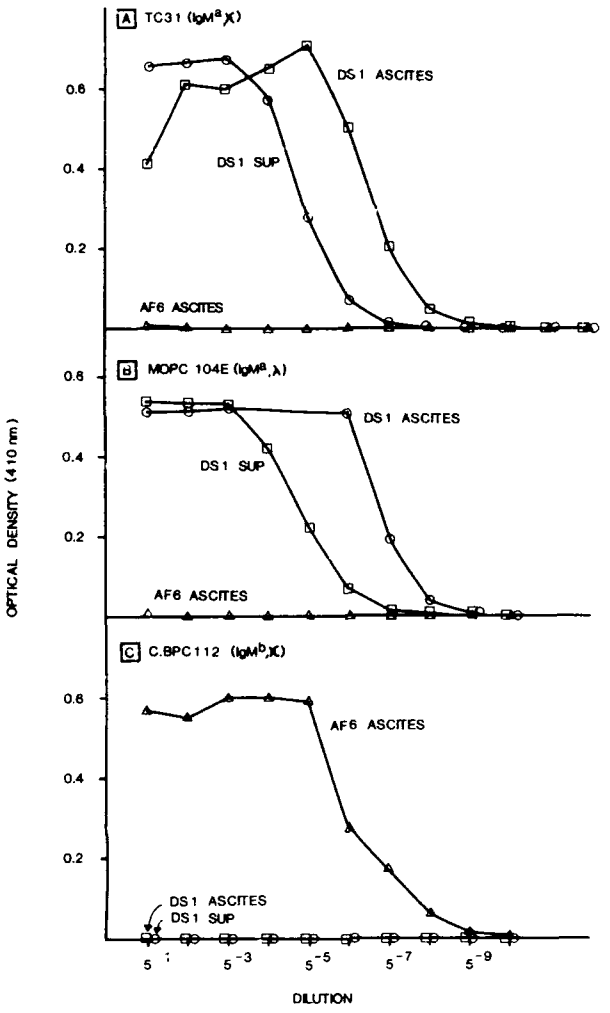

FIGURE 1. DS1 culture supernatant or DS1 ascites from CByB6F1/J mice tested in an ELISA for binding to TC31 (Panel A), MOPC104E (Panel B), and C.BPC112 (Panel C). As a control, the binding of mouse monoclonal anti-IgM^b allotype antibody, AF6-78.25, was also tested.

plates. Sera from the a, c, f, g, h, and j haplotype strains completely inhibited DS1 from binding to TC31, whereas they did not inhibit AF6-78.25 from binding to C.BPC112. In contrast, sera from the b, d, and n strains inhibited AF6-78.25 but not DS1 binding to their respective ligands on C.BPC112 or TC31. Sera from either e or o haplotypes was not able to inhibit either DS1 or AF6-78.25 binding. All sera were demonstrated to have adequate concentrations of serum IgM by their inhibition of a polyclonal goat anti-mouse μ chain antibody.

In addition, spleen cells from these same mouse strains were analyzed by FACS for binding of FL-DS1, FL-AF6-78.25, or FL-goat anti-mouse Ig. In concordance with the antigen capture and binding inhibition data, lymphocytes from Igh^a, *Igh^c*, *Igh^f*, *Igh^g*, *Igh^h* and *Igh^j* haplotypes were bound by FL-DS1, but not by FL-AF6-78.25 (Table 1). Conversely, lymphocytes from Igh^b, Igh^d, and Igh^n haplotypes, which were not stained by FL-DS1, were bound by FL-AF6-78.25. Neither FL-DS1 nor FL-AF6-78.25 were able to stain cells from *Igh^e* haplotype strain. As a control, all strains were shown to be optimally stained by a FL-goat anti-mouse Ig.

TABLE 1
Determining the Allotypic Specificity of DS1 by Antigen Capture ELISA or by Cell Surface Staining of Splenic Lymphocytes

MOUSE STRAIN	IMMUNO-GLOBULIN HAPLOTYPE	OPTICAL DENSITY[a]			%STAINING[b]		
		DS1	MOPC 245	CORRECTED DS1	FL-DS1	FL-AF6-78.25	GOAT ANTI-Ig
BALB/cByJ	a	1.88	0.40	1.48	54.9	1.6	64.4
NMRI	a	1.25	0.24	1.01	37.6	0.8	60.5
C57BL/6	b	0.16	0.15	0.00	0.2	52.5	64.6
C57BL/10SnJ	b	0.15	0.21	0.00	n.d.	n.d.	n.d.
C.B20	b	0.44	0.47	0.00	1.4	37.4	48.5
SJL/J	b	0.23	0.36	0.00	1.3	43.8	61.8
DBA/2J	c	1.42	0.40	1.02	55.6	0.9	67.0
AKR/J	d	0.22	0.30	0.00	1.2	54.5	49.7
A/J	e	0.15	0.20	0.00	1.1	1.4	57.7
CE/J	f	1.65	0.38	1.27	54.2	1.4	66.5
RIIIs/J	g	1.25	0.12	1.13	43.8	1.9	58.9
SEA/GnJ	h	1.55	0.56	0.99	67.7	0.8	51.0
CBA/J	j	1.92	0.39	1.53	42.4	1.9	56.8
NZB/BlNJ	n	0.72	0.73	0.00	1.5	33.0	54.2
AL/NIH	o	0.12	0.12	0.00	n.d.	n.d.	n.d.

[a] 100 μl of a 1:10 dilution of normal mouse serum was incubated on ELISA plates coated with either 1μg/ml purified DS1 or MOPC245 followed by an AP-conjugated goat anti-IgM antibody. The column labled "corrected DS1" is the calculated OD for binding of the serum to DS1 minus the OD for binding of the serum to the MOPC245 control plate.

[b] % positively stained cells in channels 350-1000, after subtraction of control staining by FL-MOPC245 within the same channels. n.d.= not determined.

In addition, the allotypic specificity of DS1 for IgM^a and IgM^b allotype determinants was directly compared with the specificity shown by previously described anti-IgM^a allotype MAb, namely BET 1 and 331.12. Fig. 2 shows that each MAb manifests a unique binding specificity in diferentiating the a and b allotypic diferences between TC31 and CBPC.112, respectively. DS1 clearly shows complete lack of binding to the b allotype, whereas BET 1 shows a partial binding to the b allotype, and 331,12 binds to both a and b allotypes almost equally, similar to BET 2.

Binding of DS1 to Surface Membrane of B Lymphocytes

In order to test for the reactivity of DS1 for membrane bound IgM on B lymphocytes, spleen cells from BALB/cByJ and C57BL/6J were incubated with various concentrations of a fluorescein-conjugated DS1 (FL-DS1) as described in the Materials and Methods section. Cells were also incubated with a fluorescein-conjugated AF6-78.25 (FL-AF6-78.25) or MOPC245 (FL-MOPC245) as an isotype control. Histograms of the analysis of these cell preparations on the FACS (Fig. 3, Panels A-D) show that FL-DS1 bound to splenic B cells in a

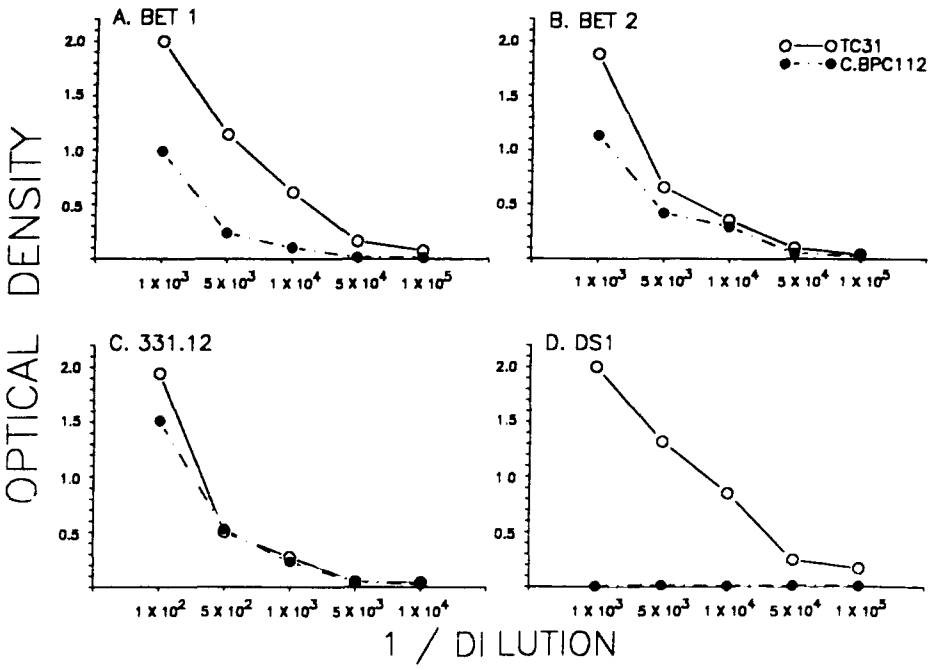

FIGURE 2. Binding of purified preparations of DS1 (3.56 mg/ml), BET 1 (2.89 μg/ml), and BET 2 (13.4 mg/ml) and culture supernatant of 331.12 to TC31 or C.BPC112 plates. Binding was detected with biotin-labled MAR18.5 (Mouse anti-rat kappa MAb), followed by AP-conjugated avidin.

strain-specitic manner. Panel A shows the binding of BALB/c cells with FL-DS1 and FL-MOPC245. Integration of the curves showed that FL-DS1 bound 50.7% of the BALB/c spleen cells, while the control antibody, FL-MOPC245, bound only 2.2% of the cells. Using C57BL/6 spleen cells, only 1.9% of the cells were bound by FL-DS1 and 1.7% by FL-MOPC245 (Panel C). In contrast, Panel B shows that FL-AF6-78.25 bound only 3.3% of the BALB/c cells, while it bound 54.4% of the C57BL/6 cells (Panel D). Binding of DS1 to BALB/c lymphocytes could be completely blocked by prior incubation of the cells with non-fluoresceinated DS1 (data not shown).

Induction of In Vitro Proliferation of Allotype Specific B Lymphocytes

The allotypic specificity of DS1 was further demonstrated by use of DS1 conjugated Sepharose to stimulate proliferation of mouse spleen cells bearing the appropriate allotypic specificity (Table 2). DS1-conjugated Sepharose beads induced significant proliferation at 48 h in cultures of BALB/cByJ and (C57BL/6 x DBA/2J)F1 mice, but was not mitogenic for C57BL/6 or AL/N (IgM^e) spleen cells. In contrast, AF6-78.25-conjugated Sepharose stimulated C57BL/6 and (C57BL/6 x DBA/2)F1 spleen cells, but not BALB/cByJ or AL/N. These data demonstrate the allotypic specificity of these MAb in binding membrane associated μ chains and also demonstrate their ability to induce proliferation of B lymphocytes in vitro, as was previously shown using soluble polyclonal goat anti-mouse μ chain antibodies (24) or rabbit anti-mouse Ig antibodies conjugated to beads (26).

Demonstration of Linkage to the Igh-C Gene Complex

To further determine the specificity of the DS1 antibody, it was important to test for linkage of the determinant recognized by DS1 to the *Igh-C* complex, which encodes the constant regions of the heavy chains of mouse immunoglobulins. Sera

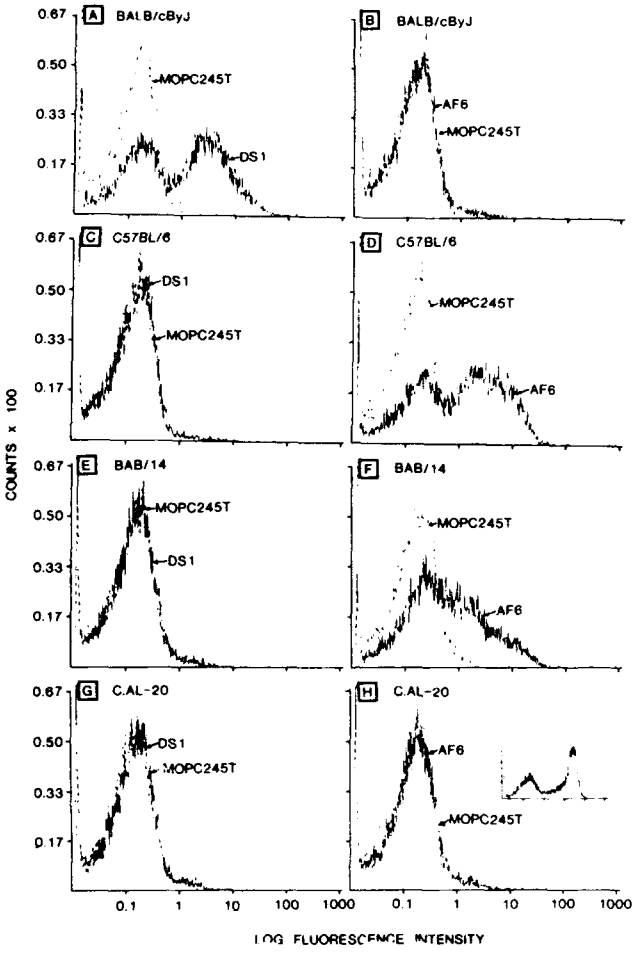

FIGURE 3. FACS analysis for binding of Fl-DS1, Fl-AF6-78.25, or control antibody, Fl-MOPC245 to membrane IgM on spleen cells from BALB/cByJ (Panels A and B), C57BL/6J (Panels C and D), BAB/14 (Panels E and F), or C.AL-20 (Panels G and H) mice. The insert graph in panel H shows control staining of 60.8 % of the C.AL-20 spleen cells by a Fl-conjugated goat anti-mouse Ig reagent.

from parental mouse strains and strains congenic at the *Igh-C* locus were used to inhibit the binding of DS1 or goat anti-μ chain antibody in an ELISA on plates coated with TC31. As a control, the sera were also tested for their ability to inhibit the binding of AF6-78.25 to C.BPC112 coated plates. The results in Table 3 demonstrate that the sera from all congenic strains gave inhibition patterns, consistent with their Ig allotype. Sera from C.B20 and BAB/14 strains, which have a BALB/c genetic background, but carry the *Igh-C*^{b} locus derived from C57Bl/6, were able to inhibit AF6-78.25, but not DS1 binding. Serum from C.AL20, which has a BALB/c background, but an *Igh-C*^{o} locus (with the *Igh-6*^{e} allele), resembled serum from the parental IgM^e allotype expressing AL/N strain, in that it was not able to significantly inhibit either DS1 or AF6-78.25 binding. In contrast, both AL/N and C.AL-20 sera contained sufficient IgM to inhibit the goat anti-μ reagent.

The above data were confirmed by FACS analysis of BAB/14 and C.AL-20 spleen cells as shown in Fig. 3 (Panels E-H). BAB/14 spleen cells were bound by FL-AF6-78.25 (Panel F, 35.5% positive), but not by FL-DS1 (Panel E, 0.3% positive), when the curves are compared with spleen cells which had been

TABLE 2
Stimulation of Allotype Specific B Cells to Proliferate In Vitro
by Anti-Allotypic Monoclonal Antibodies[a]

MITOGEN	C57BL/6J IgM^b	BALB/CByJ IgM^a	B6D2F1 $IgM^b \times IgM^a$	AL/N IgM^e
		CPM PER CULTURE		
MEDIUM	4,293	4,845	6,204	5,518
GOAT ANTI-μ BEAD	102,177	95,639	112,003	38,103
ANTI-IGM^a BEAD	4,003	66,693	79,355	4,966
ANTI-IGM^b BEAD	38,405	3,527	35,722	5,050
MOPC245 BEAD	3,876	4,106	6,644	4,910
MOPC21 BEAD	3,021	2,790	5,146	3,801

[a] Cultures of 3×10^5 spleen cells were cultured with the indicated mitogens for 48 h and then pulsed with ^{3}H-thymidine for an additional 16 h. Data represent the geometric mean of triplicate cultures.

incubated with FL-MOPC245T control IgG_1 antibody. Panels G and H show that C.AL20 cells were not bound by either FL-DS1 or FL-AF6-78.25.

The following experiments further demonstrate that the determinant on IgM which is recognized by DS1 is linked to the *Igh-C* locus. First, the progeny of an (*Igh-6*^{a} haplotype x *Igh-6*^{b} haplotype)F1 strain that was backcrossed to the b parent, were examined for the ability of their sera to inhibit the binding of DS1 to TC31. In this experiment the backcross progeny of (BALB/c x C57BL/6)F1 x C57BL/6 consisted of 38.6% (17/44) which were non-ractive with DS1 and 61.4% (27/44) which were reactive with DS1. These data are within the 95% confidence limits for the predicted Mendelian ratio of 1:1 for the backcross of the F1 to the parent.

TABLE 3
Inhibition of the Binding of DS1, AF6-78.25, or Goat Anti-μ Chain Antibodies
by Serum of *Igh-C* Congenic Mouse Strains

Serum	Inbred Back-ground	Source of *Igh-C* locus	Immuno-globulin haplotype	DS1	AF6-78.25	Goat anti-μ
				% INHIBITION[a]		
BALB/cByJ			a	97	0	85
C57BL/6			b	9	36	77
AL/N			o	8	3	73
C.B-20	BALB/c	C57BL/Ka	b	6	71	87
BAB/14	BALB/c	C57BL/Ka	b	12	77	87
C.AL-20	BALB/c	AL/N	o	1	0	79
FCS				3	0	0

[a] The displayed values are for the sera diluted 1:100 before addition to a equal volume of ascites diluted at 1:1500 or goat anti-μ at 1:5000. The optical densities for the binding of the antibodies to the plates without inhibitors (to be used as 100% control values) were: 0.694 and 0.660 for DS1, 0.534 and 0.5?i for AF6-78.25, and 0.480 and 0.520 for goat anti-μ antibody.

TABLE 4

Typing of Serum IgM From Recombinant Inbred Lines[a]

AKXL LINES					BXD LINES				
		% INHIBITION					% INHIBITION		
RI LINE	*Igh-1* ALLELE[b]	DS1	AF6	*Igh-6* ALLELE	RI LINE	*Igh-1* ALLELE[b]	DS1	AF6	*Igh-6* ALLELE[b]
6	a	95	0	a	2	b	27	99	b
7	-	92	64[c]	a	5	b	12	95	b
8	d	0	99	b	6	c	100	0	a
9	-	8	89	b	8	b	7	100	b
12	d	3	97	b	9	b	99	4	a
13	a	98	8	a	11	c	100	15	a
16	a	97	16	a	15	c	100	11	a
17	d	0	100	b	16	c	98	12	a
19	a	100	16	a	17	c	100	21	a
21	a	98	15	a	18	c	99	11	a
28	d	1	98	b	19	b	9	95	b
29	a	92	13	a	20	c	99	11	a
37	d	0	94	b	24	c	99	7	a
38	d	3	97	b	27	c	100	19	a
					28	c	99	0	a
					29	b	3	98	b
					30	c	100	2	a

[a] Sera were used at a 1:10 dilution for dilution with an equal volume of DS1 or AF6-78.25 ascites at 1:1500. Control OD for the binding of MAb without inhibitors (for the 100% control) were: 0.772, 0.810, and 0.652 for DS1 binding and 0.562, 0.560, and 0.521 for AF6-78.25 binding.

[b] From ref. 26.

[c] At 1:100 serum dilution only 1% of AF6-78.25 binding was inhibited.

In another experiment to test linkage, sera from recombinant inbred (RI) strains were typed for the *Igh-6* haplotype using the DS1 and AF6-78.25 monoclonals. The AKXL and BXD lines were derived by Taylor from the crosses of AKR/J (*Igh-C*^{d}, *Igh-6*^{b}) with C57L/J (*Igh-C*^{a}, *Igh-6*^{a}) and of C57BL/6J with DBA/2J *Igh-C*^{c}, *Igh-6*^{a}), respectively, by brother-sister mating of the F2 generation progeny (27,28). These strains have previously been typed for the *Igh-1* locus by Taylor and colleagues (29). The data in Table 4 show the ability of sera from the RI lines to inhibit either DS1 or AF6-78.25 monoclonal from binding to their respective myeloma carrying the a or b allotype. Based on the inhibition data, the *Igh-6* haplotype of each RI line was assessed. In each case, the *Igh-6* locus appeared to be linked to the *Igh-1* locus. In the AKXL lines, the *Igh-6*^{a} allele was always found in strains displaying the *Igh-1*^{a} allele, and similarly, the *Igh-6*^{b} allele was always found in strains carrying the *Igh-1*^{d} allele. Similar conclusions can be made for the BXD lines, where there is 100% coorelation for the expression of Igh-1 and Igh-6 alleles. Thus these data clearly demonstrate that the determinant recognized by DS1 is linked to the Igh-C locus.

Localization of the Allotypic Determinant Recognized by DS1 to a Specific Heavy chain domain

Localization of the heavy chain domain bound by DS1 was determined by use of monoclonal rat anti-μ chain antibodies which have been shown to bind to either the CH1, CH2, or CH3 domains of the μ heavy chain (18). These and other rat anti-μ chain antibodies were used to inhibit the binding of DS1 in the ELISA to IgM^a (TC31). The results of these experiments (Table 5) suggest that the epitope recognized by DS1 is located in the CH1 domain. The MAb specific for the CH1 domain, R33, and Ber 1, strongly inhibited the binding of DS1 (approx. 70%), while those antibodies which reacted with the CH2 or CH3 domains (B76, IM41, and

TABLE 5
Inhibition of Binding of DS1 by Monoclonal Rat Anti-mouse IgMs

Rat Monoclonal Anti-μ Antibody	Heavy Chain Constant Domain Specificity	% Inhibition of DS1 Binding[a]	Antibody[b] Binding to TC31 (Optical Density)
R33	CH1	71	0.620
B76	CH2	16	1.145
IM41	CH3	13	0.579
2911	CH3	6	0.534
331.12	CH1	0[c]	0.432
Bet 1	CH1	69	0.657
Bet 2	CH2	17	0.519

[a] Rat anti-μ hybridoma supernatant was incubated with TC31 plates for 2 h before addition of a 1:1500 dilution of DS1 ascites. Binding of DS1 was detected with an AP-conjugated goat anti-mouse IgG, Fc specific antibody. 100% binding of the DS1 without inhibitors gave an OD of 1.189.

[b] Binding of rat anti-μ supernatant to TC31 coated plates was detected using a goat anti-rat IgG antibody followed by an AP-conjugated swine anti-goat IgG.

[c] An additional experiment using purified 331.12 MAb showed at least a 100-fold less inhibition by 331.12 than by DS1 in inhibition of DS1 binding to TC31.

2911) showed little or no inhibition of DS1. Two other μ chain specific MAbs (Bet 2 and Bet 3) showed little or no inhibition of DS1. These data suggest that the allotypic determinant recognized by DS1 resides in the CH1 domain of the μ heavy chain. Furthermore, we have found that the staining of spleen cells with DS1 antibody can be inhibited by mutant IgM proteins that lack CH2, CH3, or CH4 deomains, but not by IgM molecules that are deficient in the CH1 domain (B. Subbarao, unpublished data).

Interestingly, 331.12 and Bet 1, which have similar (though not identical) IgM allotypic reactivity, differ markedly in their ability to inhibit DS1 binding (Table 5). These antibodies both recognize the a and b but not the e allele of IgM, however, they differ in the level of reactivity to the b allele (Fig. 2, Table 6, ref. 9, 10, A. Stall, unpublished observation). Immuno-

TABLE 6
The *Igh-6* Locus

			IgM ALLOTYPIC SPECIFICITIES								
			6.1			6.2	6.3	6.4	6.5		6.6
Igh-C HAPLOTYPE	*Igh-6* ALLELE	TYPE STRAIN	*Monoclonal or Serum (Reference)* MA-3[a] (7)	RS-3.1 (14)	DS1	GA-20[a] (7)	MB-3[a] (7)	MB86 (13)	BET-1 (11)	331.12 (9,10)	AF6-78.25 (12)
a	a	BALB/c	+	+	+	+	-	-	+	+	-
b	b	C57BL/6	-	-	-	-	+	+	+(wk)	+	+
c	a	DBA/2		+	+			-	+	+	-
d	b	AKR		-	-			+	+(wk)	+	+
e	e	A		-	-			+(wk)	-	-	-
f	a	CE			+						-
g	a	RIIIs			+						-
h	a	SEA/Gn			+						-
j	a	CBA		+	+			-	+	+	-
n	n	NZB	-	-	-	+	-	+	+(wk)	+	+
o	e	AL/N			-			+(wk)	-	-	-

[a] Anti-allotypic antisera generated by S. Black, et al. (8).

fluorescence experiments with 331.12 and the IgM domain loss mutants have shown that this MAb also binds to an epitope on the CH1 domain (V. Udhyakumar and B. Subbarao, manuscript in preparation). Since these three antibodies, Bet 1, 331.12, and DS1 all bind to the CH1 domain but have unique reactivities with the IgM proteins from the a and b allotype mice, they must bind to different epitopes on the CH1 domain.

DISCUSSION

This communication describes a new mouse monoclonal antibody with specificity for the *Igh-6*^{a} allele of mouse IgM immunoglobulin. Anti-allotype antisera for mouse IgM have been difficult to prepare and have only been reported to have been generated by immunization of mice with a polyclonal IgM as a pertussis-anti-pertussus complex or by immunizations with spleen cells in mice which differ in H-2 and allotype (7,8). Monoclonal anti-allotype reagents have been obtained by immunizing with spleen cells (13), by immunizing across species barriers to develop rat monoclonal (10,11), or most recently by immunizing mice with polyclonal serum IgM (15). It is of interest to note that the anti-IgM^a monoclonal described in this paper was derived from C57BL/6 mice immunized with a monoclonal IgM antibody. The TC31 IgM monoclonal which was used for immunization was developed from the NMRI strain of mouse, which is an inbred line derived from Swiss outbred mice. Its IgG immunoglobulins have been typed as having the *Igh-C*^{a} haplotype (Dr. R. Lieberman, personal communication). It is not known whether the immunizing IgM monoclonal had a unique structure which made recognition of the allotype possible using this immunization scheme. Using a sensitive enzyme-linked-immunoassay, anti-allotypic antibody could be detected in the serum of the immunized mice. Only one hybridoma producing anti-allotypic activity was detected in this fusion involving the screening of nearly 500 wells.

The allotypic specificity of the DS1 monoclonal was confirmed by a) the ability to bind to other BALB/c derived myeloma IgM proteins of different idiotypic specificities that were derived from BALB/c, b) the inability to bind to an IgM myeloma protein which was derived from C57BL/6, c) the inhibition of the MAb by serum from BALB/c, but not C67BL/6, and d) the ability of DS1 to induce proliferation of IgM^a bearing spleen cells. These data demonstrated that DS1 was not an anti-idiotypic or anti-isotypic antibody.

The allotypic specificity which is recognized by DS1 was demonstrated to be expressed in strains of *Igh-C* haplotype a, c, f, g, h and j, and not expressed in strains of *Igh-C* haplotype b, d, e, n and o. A similar pattern of reactivity has been previously identified by an anti-allotypic antiserum, MA-3, derived by Black and colleagues (8) and designated by them as specificity Igh-6.1. The MA-3 antiserum reacted with serum from BALB/c (*Igh*^{a}), but not with C57BL/6 (*Igh*^{b}), NZB (*Igh*^{n}), or allotype congenic strains bearing either the b or e alleles. The antigenic specificities of the *Igh-6* locus and a summary of the reactivities of the previously defined antisera and monoclonal antibodies is summarized in Table 6. Since the reactivity of DS1 is indistinguishable from the published definition of 6.1, we propose that DS1 be designated as being specific for Igh-6.1. The reactivity of DS1 is different from the published specificity of rat monoclonal Bet 1, which, in addition to reacting with the specificities seen by DS1, also displays a weak reaction with haplotypes b, d and n (12). These differences in reactivity may be due to the recognition by the rat monoclonal of additional species-specific determinants which are common to both the a, b and n haplotypes.

Linkage of the determinant recognized by DS1 to the *Igh-C* locus was demonstrated by a) the inability of DS1 antibody to bind to serum IgM from allotype congenic strains, C.B20, BAB/14, and C.AL-20, which have the BALB/c background, but have an *Igh-C* locus from another strain, b) the inability of FL-DS1 to stain membrane IgM from the allotype congenic strains, c) segregation of the antigenic determinant recognized by DS1 together with the *Igh-1*^{a} determinant in a series of recombinant inbred lines of AKXL and BXD mice, and d) a Mendelian 1:1 ratio for the segregation of the allotypic marker in (BALB/cByJ x C57BL/6J)F1 x C57BL/6J backcross mice. In addition, by using a series of rat anti-μ chain monoclonals which have been defined in terms of their specificity for the four constant region domains of the μ chain (18), the antigenic

determinant recognized by DS1 was localized to the first constant domain (CH1). These data are consistent with the recent report of Schrier and colleagues (30), who sequenced the c-DNA of C57BL/6 μ heavy chain and compared it with sequences of DNA for BALB/c μ chains. They found seven nucleotide differences between μ chain sequences in the two strains; however, only one of the nucleotide differences was predicted to lead to an amino acid change. They concluded that a single amino acid change was the molecular basis of the allotypic differences between the a and the b alleles for the μ chain. This amino acid difference occurs at codon 222 (in the OU numbering system) and involves a lysine residue in the b allele and an arginine in the a allele. Amino acid residue 222 has been defined to be in the CH1 domain of mouse μ chain (31).

DS1, Bet 1 and 331.12 each have a unique reactivity with the two alleles of IgM. DS1 reacts with IgM^a, but not with IgM^b, Bet 1 reacts strongly with IgM^a, but weakly with IgM^b (12), while 331.12 reacts equally well with both alleles (11, Fig.3, Table 6). None of the antibodies recognize the IgM^e allele. In addition, the epitopes recognized by DS1 and 331.12 must be spatially distinct since the antibodies do not cross block. In contrast, the epitopes recognized by DS1 and Bet 1 must overlap, since Bet 1 can block DS1 binding (Table 5). This indicates that these three antibodies define three individual allotypic epitopes that differ between the IgM^a and IgM^b alleles. Thus the anti-allotypic MAb reveal a serological complexity greater than that suggested by the single amino acid difference between the a and b alleles of IgM. It is probable that the single amino acid difference induces conformational changes which can generate more than one serological epitope. This idea is consistent with the studies of Velardi, et al. (11) demonstrating that, in contrast to class specific antibodies, anti-IgM allotypic antibodies (both anti-IgM^a and anti-IgM^b) can react with intact IgM but cannot react with free μ heavy chain, indicating that IgM allotypic epitopes are generated from the conformational interactions of both the heavy and light chains.

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